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**COMPOSITION AND DEVICE FOR DETECTING LEUKOCYTES  
IN URINE**

**Field of the Invention**

10        This invention relates generally to compounds, compositions, devices, and methods useful for detecting the presence of leukocytes through the activity of leukocyte esterases and proteinases in urine.

**Background of the Invention**

15        The presence of leukocytes in human urine is associated with infection or malfunction in the kidney or urinary tract. Accurate detection has a significant meaning for the physiological treatment or diagnosis of the patient.

20        A basic method to measure the number of leukocytes in urine by microscope has been widely available for some time. However, the disadvantages of this method include the investment of time and money in obtaining and installing the appropriate instrumentation. Further, false negatives can be obtained when samples are allowed to sit too long before analysis.

25        Research has been directed to developing other methods, such as indicator assays, that are suitable for detecting leukocytes more easily, conveniently, and accurately. Typical indicator assays use a specific chemical substance (e.g., a substrate) that is degraded by one or more enzymes present in leukocytes to create a product suitable for effectuating a visible color change.

30        One such leukocyte assay is disclosed in U.S. Patent No. 3,087,794 and involves the use of peroxidase that is contained in a granular leukocyte. This assay includes a filter paper stained with hydrogen peroxide and *o*-tolidine, which shows a colored oxidative product when contacted with leukocytes. Yet this assay is less than desirable because peroxidase can be dangerous and reductive materials in urine may make actual application impractical.

35        Other methods designed to confirm the presence of esterase and proteinase in leukocytes have also been developed. For example, one method uses a colorless or

pale-colored ester compound as a substrate that is degraded by esterase into a colorless acid moiety and an alcoholic moiety. Then, under diazonium or oxidative reaction, the alcoholic moiety is converted to a second densely colored product. This method was derived from a process in which enzymatic degradation of the substrate naphtol-AS-D  
5 chloroacetate produced chloroacetate and naphtol-AS. Reaction of the naphtol-AS product with a diazonium salt resulted in the formation of a colored azo compound.

This assay allows for the determination of leukocyte concentration by the naked eye. Yet the use of this assay is less than desirable because the diazonium salt may react with urobilinogen or bilirubin contained in urine. As a result, concentrations of  
10 leukocytes greater than 500 cells/ $\mu$ l are often necessary to avoid a false-negative reading.

U.K. Patent No. 1,128,371 discloses other possible substrates, i.e., colorless indoxyl or thioindoxylesters. These substrates are degraded into indoxyl or thioindoxyl by esterase. Colored indigo or thioindigo may then be produced by reaction with  
15 oxygen in the air or by an oxidizer. Yet the use of an assay with these substrates is less than desirable because this is not sensitive and fails to detect leukocytes at a concentration less than 10,000 cells/ $\mu$ l.

Similarly, U.S. Patent No. 4,278,763 suggests indoxyl-type substrates by disclosing a method of using indoxyl or thioindoxylamino acid ester as a substrate.  
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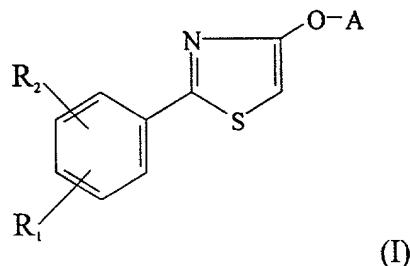
Other assays using pyrrole derivatives as substrates have also been disclosed. For example, U.S. Patent No. 4,704,460 discloses use of an amino acid ester of a pyrrole derivative as a substrate. When this derivative is degraded in the presence of diazonium salt, a change in sample color to deep violet results. Moreover, the addition of a nucleophilic alcohol, such as decanol, greatly facilitates reaction rate, and in turn, allows  
25 detection of leukocyte concentrations as low as 10 cells/ $\mu$ l within 90 seconds. Yet this assay is less than desirable because the syntheses of these two substrates is very difficult.

The above-described assays are characterized by numerous disadvantages. Some disadvantages include the detection of false negatives, the interference of  
30 urobilinogen or bilirubin, the lack of sensitivity, and the requirement of less-than-desirable substrates. Thus, it would be desirable to identify new compounds and methods of using the compounds to detect the presence of leukocytes in urine.

Summary of the Invention

The invention is directed to thiazole esters suitable for detecting leukocytes in urine, compositions containing thiazole esters, diagnostic devices suitable for detecting leukocytes in urine, and methods of using the thiazole esters or compositions thereof for detecting leukocytes in urine.

In one aspect, this invention is directed to a novel thiazole ester. A thiazole ester of the invention is of the formula:



or a salt or solvated salt thereof, in which

A is an N-blocked amino acid residue or an N-blocked peptide chain, preferably N-blocked alanine or N-blocked polyalanine; and

15 R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted alkyl, unsubstituted or substituted alkenyl, unsubstituted or substituted alkoxy, amino, unsubstituted or substituted acyl, halo, nitro, cyano, -SO<sub>3</sub>H, or hydroxy, with the proviso that R<sub>1</sub> and R<sub>2</sub> are not both hydrogen.

20 In another aspect, this invention is directed to compositions that include a thiazole ester of formula I. Compositions of the invention may also include a diazonium salt such as 2-methoxy-4-morpholinobenzene diazonium chloride, zinc chloride double salt.

25 In still another aspect, this invention is directed to diagnostic devices that include a compound or composition of the invention. Such diagnostic devices include a substrate having a thiazole ester of formula I deposited thereon.

The compounds, compositions, and diagnostic devices of the invention are also suitable for use in methods for detecting leukocytes in urine. Methods for detecting

leukocytes in urine include contacting a thiazole ester of the invention and a diazonium salt with a urine sample.

The compounds and compositions suitable for use in devices and methods of the invention are typically pharmaceutically acceptable.

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#### DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to detecting leukocytes in urine. The presence of leukocytes is typically detected by detecting the presence of enzymes known in the art as leukocyte esterases and leukocyte proteinases that are present with leukocytes.

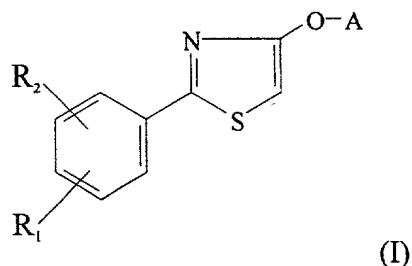
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Compounds of the invention include thiazole esters that contain an ester functionality, which is hydrolyzed upon exposure to leukocytes in urine. The hydrolysis of the ester of a thiazole ester of the invention provides a useful diagnostic technique because thiazolyl can react with a diazonium salt to produce an azo dye.

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The compounds according to this invention are thiazole esters that are suitable for use in compositions, diagnostic devices, and methods that can be used to detect leukocytes in urine.

A thiazole ester according to the invention is of the formula:



20 or a salt or solvated salt thereof, in which A preferably is an N-blocked amino acid residue or N-blocked peptide chain, preferably N-blocked alanine or N-blocked polyalanine (e.g., alanine-alanine, alanine-alanine-alanine, and the like) being blocked at the amino terminus by protecting groups known in the art, such as, for example, benzyloxycarbonyl, *t*-butoxycarbonyl, and *p*-toluenesulfonyl; and R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted alkyl, unsubstituted or substituted alkenyl, unsubstituted or substituted alkoxy, amino, unsubstituted or substituted acyl, halo, nitro, cyano, -SO<sub>3</sub>H, or hydroxy, with the proviso that R<sub>1</sub> and R<sub>2</sub> are not both hydrogen.

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Any substituted R<sub>1</sub> and R<sub>2</sub> groups may be further substituted by aryl, heteroaryl, alkyl, alkenyl, alkoxy, amino, acyl, halo, nitro, cyano, -SO<sub>3</sub>H, or hydroxy.

The term "aryl" includes aromatic hydrocarbyl, such as, for example, phenyl, including fused aromatic rings, such as, for example, naphthyl, anthryl, and the like.

5       The term "heteroaryl" includes heterocyclic aromatic derivatives having at least one heteroatom, such as, for example, nitrogen, oxygen, phosphorus, or sulfur, and includes, for example, furyl, pyrrolyl, thienyl, oxazolyl, pyridyl, imidazolyl, thiazolyl, isoxazolyl, pyrazolyl, isothiazolyl, and the like. The term "heteroaryl" also includes fused rings in which at least one ring is aromatic, such as, for example, indolyl, purinyl,  
10      benzofuryl, benzothienyl, quinolyl, 4,5,6,7-tetrahydro-1*H*-indolyl, and the like.

The term "alkyl" includes a straight or branched saturated aliphatic hydrocarbon chain having from 1 to 4 carbon atoms, such as, for example, methyl, ethyl, propyl, isopropyl (1-methylethyl), butyl, *t*-butyl (1,1-dimethylethyl), and the like.

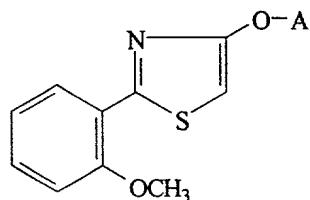
15      The term "alkenyl" includes an unsaturated aliphatic hydrocarbon chain having from 2 to 4 carbon atoms, such as, for example, ethenyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-methyl-1-propenyl, and the like.

20      The above alkyl or alkenyl groups may optionally be interrupted in the chain by a heteroatom, such as, for example, a nitrogen or oxygen atom, forming an alkylaminoalkyl or alkoxyalkyl group, for example, methylaminoethyl or methoxymethyl, and the like.

25      The term "alkoxy" includes alkyl as defined above joined to an oxygen atom having from 1 to 4 carbon atoms in a straight or branched chain, such as, for example, methoxy, ethoxy, propoxy, isopropoxy (1-methylethoxy), butoxy, *t*-butoxy (1,1-dimethylethoxy), and the like.

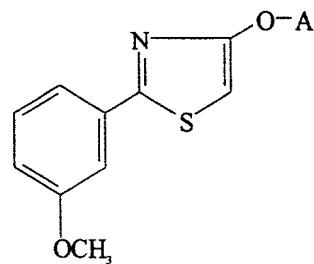
30      The term "amino" includes a substituent of the formula -N(R<sub>3</sub>)<sub>2</sub> in which each R<sub>3</sub> is independently hydrogen or alkyl. The term "alkyl" is as defined above.

In one embodiment, at least one of R<sub>1</sub> and R<sub>2</sub> is methoxy, ethoxy, propoxy, or butoxy, and A is an N-blocked alanine residue. In another embodiment, R<sub>1</sub> is hydrogen and R<sub>2</sub> is methoxy, ethoxy, propoxy, or butoxy. For example, methoxy may be substituted at the *ortho* position and the compound of formula I may be:



Alternatively, methoxy may be substituted at, for example, the *para* or the *meta* position and the compound of formula I may be:

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A composition of the invention includes a thiazole ester of formula I. Such a composition may also include a diazonium salt such as 2-methoxy-4-morpholinobenzene diazonium chloride, zinc chloride double salt.

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Compositions of the invention may be free of salts known in the art to have an accelerating action. Examples of salts that give an accelerating action include salts of monovalent and divalent cations of the alkali metals and alkaline earth metals, such as, for example, Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>++</sup> as well as the corresponding anions.

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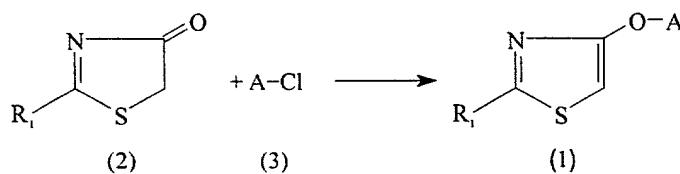
A diagnostic device suitable for detecting leukocytes in urine includes a substrate suitable for supporting a thiazole ester of the invention, such as, for example, filter paper, filtration membrane, and other inert carriers. These substrates are known in the art. The diagnostic device also includes a thiazole ester of formula I. This compound is included with the substrate by, for example, sedimenting the derivative onto the substrate.

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Generally, a novel diagnostic device may be prepared by combining the thiazole ester, an accelerator, and a diazonium salt. Accelerators suitable for use in compositions and methods of the invention are known in the art and include, for example, octanediol and decanol.

Diazonium salts suitable for use in compositions and methods of the invention are known in the art and include compounds that may be used for color-developing technology, such as, for example, 1-diazo-8-naphtol-3,6-disulfonic acid, chloride, zinc chloride double salt; 6-diazo-1-naphtol-3-sulfonic acid, chloride double salt; and 5 2-methoxy-4-morpholinobenzene diazonium chloride, zinc chloride double salt. Preferably, the diazonium salt used in accordance with the invention has no interaction with urobilinogen and/or bilirubin.

- Methods of manufacturing a device according to this invention are known in the art. Generally, a first solution containing boric acid and polyvinylpyrrolidone is 10 deposited onto a substrate, such as a filter paper. Then a second solution containing a thiazole ester of the invention and a diazonium salt, such as 2-methoxy-4-morpholinobenzene diazonium chloride, zinc chloride double salt, is deposited onto the substrate. Both solutions deposited onto the substrate may be free of a salt known in the art to have an accelerating action as described above.
- 15 To prepare a thiazole ester of the invention, a thiazolone derivative and an alanine or polyalanine derivative may be used as starting materials. Thiazolones may be synthesized by reacting carboxymethyl thiobenzimidate hydrobromide and pyridine.
- As illustrated in the following scheme, the synthesis of a thiazole ester of the invention includes the reaction of a thiazolone derivative of the general formula (2), and 20 an amino acid chloride of the general formula (3).



- Each starting material is dissolved in solvent, and the resulting solutions are 25 cooled and mixed slowly. This reaction mixture is stirred and then allowed to stand at room temperature for several hours. The reaction mixture is then washed, dried, filtered, and concentrated under reduced pressure. The solid, so formed, is dissolved in acetone and hexane is added. To remove any resulting hemisolid impurity, the reaction mixture is allowed to stand at low temperature for about 1 hour. Another portion of

hexane is added to the residue and the mixture is refrigerated for 10 hours. The desired product is filtered and dried.

Thiazolone derivatives tend to be highly reactive, resulting in dimerization or multimerization even during recrystallization. This may lead to poor yield. Thus, for 5 the current invention, pure substances are isolated from the final product of the synthesis of the thiazole ester without purifying thiazolone of the general formula (2). This method of synthesis enhances the yield of the derivatives of the invention.

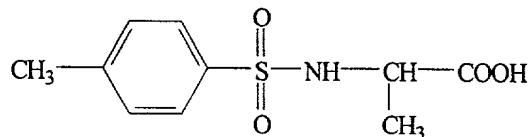
In contrast to this, the aforementioned indole or pyrrole derivatives have some recognized disadvantages. Many side reactions occur in the process of manufacturing 10 their intermediates. Moreover, the reaction mechanism is very complicated and the yield for indole and pyrrole derivatives proves to be poor. But the synthesis of the thiazole esters of this invention allows for larger amounts of final product to be produced by a general method within a relatively short period of time. Moreover, because the method of synthesizing the thiazole esters of the invention is relatively 15 simple and general, mass-scale production is more practical.

Methods of the invention include detecting the presence of leukocytes in urine. To detect the presence of leukocytes in urine, a urine sample is contacted with a thiazole ester of the invention and a diazonium salt in the presence of an accelerator. If 20 leukocytes are present, then a reaction between thiazolyl and the salt produces an azo dye having a violet color.

Typically such a method is directed to contacting a urine sample with a substrate, for example, filter paper, that has a first solution of boric acid and polyvinyl 25 pyrrolidone deposited thereon and then a second solution of a thiazole ester of formula I; a diazonium salt; and an accelerator deposited thereon. This method may be carried out free of an accelerating salt. When a urine sample containing leukocytes contacts such a substrate, then a violet color, which appears on the substrate, is observed.

#### WORKING EXAMPLES

This invention will be further characterized by the following examples. These 30 examples are not meant to limit the scope of the invention, which has been fully set forth in the foregoing description. Variations within the scope of the invention are apparent to those skilled in the art.

EXAMPLE 1N-(*p*-Toluenesulfonyl)-alanine

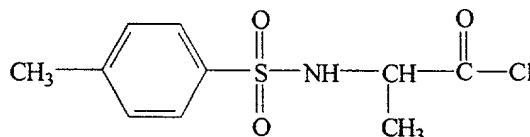
To synthesize a starting material containing a blocked N-alanine, N-(*p*-toluenesulfonyl)-alanine is first synthesized.

5      50.0 g of *p*-toluenesulfonyl chloride was added to 100 ml of 90 °C toluene with stirring. The *p*-toluenesulfonyl chloride solution in toluene was slowly added to 25.0 g of L-alanine dissolved in 500 ml of 1N NaOH cooled to 5 °C and stirred for 24 hours. The resulting aqueous layer is separated and cooled to below 5 °C and with the addition 10 of concentrated hydrochloric acid, pH was adjusted to 1.5. After standing in the refrigerator for 3 to 4 hours, the white solid crystals, so formed, were filtered, twice washed with water, and dried.

EXAMPLE 2

N-Tosyl-L-alanyl chloride

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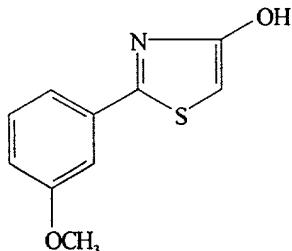


The blocked starting material is next produced by reacting N-(*p*-toluenesulfonyl)-alanine and oxalyl chloride.

20      30.0 g of N-(*p*-toluenesulfonyl)-alanine and 6 to 7 drops of DMF (dimethylformamide) were dissolved in 100 ml of dichloromethane and 5 ml of oxalyl chloride was added dropwise. After stirring for 3 hours at room temperature the reaction mixture was evaporated down to an oily residue which was dissolved in 100 ml of dichloromethane. The dichloromethane was again evaporated and the resulting 25 residue dissolved in 50 ml of dichloromethane followed by the addition of 100 ml hexane. After standing in the refrigerator overnight the crystals, so formed, were filtered and dried.

EXAMPLE 3

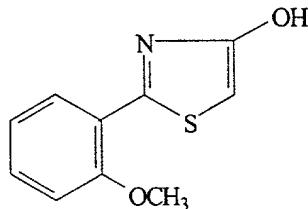
2-(3-Methoxyphenyl)-4-thiazol



5 HCl gas was bubbled through a solution of 11 g of 3-methoxybenzonitrile and 10 g of mercaptoacetic acid in approximately 75 ml of ether for 24 hours. The crystallized product, so formed, was filtered, washed with 200 ml ether, and dried.

EXAMPLE 4

10 2-(2-Methoxyphenyl)-4-thiazol

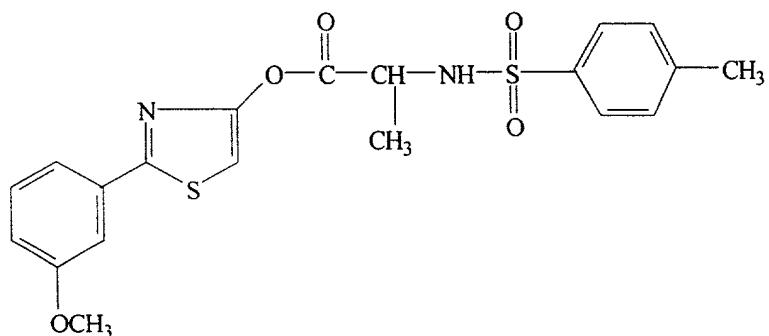


Using the method of Example 3, 2-methoxybenzonitrile and mercaptoacetic acid were mixed. The recovered product is shown above.

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EXAMPLE 5

2-(3-Methoxyphenyl)-4-(N-tosyl-L-alanyloxy) thiazol



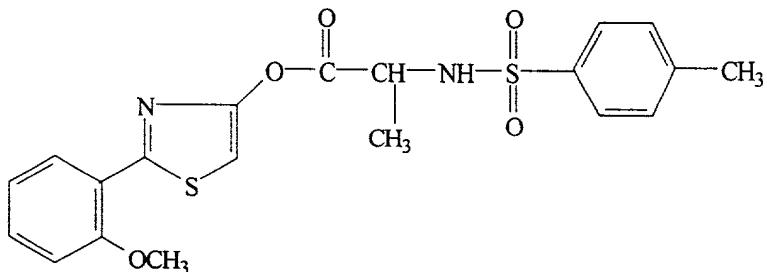
5.0 g of 2-(3-methoxyphenyl)-4-thiazol was dissolved in a cosolvent of 100 ml of dichloromethane and 10 ml pyridine and cooled to 5 °C. A solution of 15.0 g of N-tosyl-L-alanyl chloride in dichloromethane was then added dropwise to the above mixture. The reaction solution was stirred at room temperature until the reaction was  
5 complete. The solution was washed with 1N hydrochloric acid, water, saturated sodium bicarbonate, and a saturated sodium chloride solution in that order; dried over magnesium sulfonate; filtered; and concentrated under reduced pressure. The residue, so formed, was dissolved in 100 ml of acetone and 100 ml of hexane were added. After standing in the refrigerator for 1 hour, dark semisolid impurities were decanted off and  
10 the remaining solvent evaporated. The amorphous solid, so formed, was dissolved in a minimum of ethylacetate, the resulting solution applied to a silica column and eluted with a 2:1 ethylacetate/hexane solvent system. Fractions containing the desired product were collected, the solvent was evaporated, and the resulting residue was dissolved in 20 ml of acetone followed by the addition of 50 ml hexane. After standing in the  
15 refrigerator the white crystals, so formed, were filtered and dried.

The product was identified by proton NMR analysis by dissolving a sample of the product in acetone-*d*<sub>6</sub> (15mg/0.7 ml) and collecting a proton spectrum following AMRI SOP INS-041 using a Bruker® 300 MHz NMR Spectrometer (Bruker-Physik AG, Germany).

20 The identity of the product was further confirmed by infrared spectral analysis by first mixing 204 mg of potassium bromide with 1.3 mg of the product and then grinding them in a Wig L Bug® (Crescent Dental Manufacturing Co., Illinois). The ground mixture was pressed into a disk and an infrared spectrum was collected by a Spectrum 1000 IR Spectrometer following AMRI SOP INS-026. The sample was  
25 scanned between 4000 and 400 cm<sup>-1</sup>.

EXAMPLE 6

2-(2-Methoxyphenyl)-4-(N-tosyl-L-alanyloxy) thiazol



- 5        5.0 g of 2-(2-methoxyphenyl)-4-thiazol was dissolved in a cosolvent of 100 ml of dichloromethane and 10 ml pyridine and cooled to 5 °C. A solution of 15.0 g of N-tosyl-L-alanyl chloride in dichloromethane was then added dropwise to the above mixture. The reaction solution was stirred at room temperature for four hours or until the reaction was complete. The solution was washed with 1N hydrochloric acid, water,
- 10      saturated sodium bicarbonate, and a saturated sodium chloride solution in that order; dried over magnesium sulfonate; filtered; and concentrated under reduced pressure. The residue, so formed, was dissolved in 100 ml of acetone and 100 ml of hexane were added. After standing in the refrigerator for 1 hour, dark semisolid impurities were decanted off and the remaining solvent evaporated. The amorphous solid, so formed,
- 15      was dissolved in a minimum of ethylacetate, the resulting solution applied to a silica column and eluted with a 2:1 ethylacetate/hexane solvent system. Fractions containing the desired product were collected, the solvent evaporated and the resulting residue was dissolved in 20 ml of acetone followed by the addition of 50 ml hexane. After standing in the refrigerator the white crystals, so formed, were filtered and dried.
- 20      The product was identified by two proton NMR analyses. For the first analysis, a sample of the product was dissolved in acetone-*d*<sub>6</sub> (11mg/0.7 ml) and a proton spectrum was collected following AMRI SOP INS-041 using a Bruker® 300 MHz NMR Spectrometer (Bruker-Physik AG, Germany). For the second analysis, a proton spectrum was collected using a Bruker® 500 MHz NMR Spectrometer.
- 25      The identity of the product was further confirmed by infrared spectral analysis by first mixing 235 mg of potassium bromide with 2.4 mg of the product and then grinding them in a Wig L Bug® (Crescent Dental Manufacturing Co., Illinois). The

ground mixture was pressed into a disk and an infrared spectrum was collected by a Spectrum 1000 IR Spectrometer following AMRI SOP INS-026. The sample was scanned between 4000 and 400 cm<sup>-1</sup>.

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### EXAMPLE 7

#### Preparation of a Leukocyte Diagnostic Device

To detect the presence of leukocytes in urine, a test strip containing a compound of the invention was prepared. A small type of testing paper in a regular square was attached to the end of a polystyrene strip, sedimented, and dried with the following two mixing solutions successively. The first solution (100 ml of aqueous solution) contained 5% (w/v) boric acid (pH 7.7) and 2% (w/v) polyvinyl pyrrolidone (K-10). The sedimented paper was dried by heating at 60° C for 10 minutes. A second solution (100 ml of acetone) contained 0.06% (w/v) of 2-(3-methoxyphenyl)-4-(N-tosyl-L-alanyloxy) thiazole or 2-(2-methoxyphenyl)-4-(N-tosyl-L-alanyloxy) thiazole; 0.05% (w/v) 2-methoxy-4-morpholinobenzene diazonium chloride zinc chloride disalts and 1.0% (w/v) n-decanol. The sedimented paper was dried by heating at 50° C for 5 minutes.

Successful leukocyte assays (i.e., the assays were useful for correctly identifying the presence of leukocytes in urine) were carried out using a diagnostic device that contained each one of the thiazole esters listed in this Example.

Each assay was conducted by contacting a test strip (manufactured as described above) with a urine sample. When the urine sample contained leukocytes, the test strip showed the appearance of a violet color. The initial tint of violet color appeared within about 10 seconds and gradually darkened until the reaction was complete, which was about 1 minute. The completed reaction showed a degree of color change that was suitable not only for identifying whether leukocytes were present in the urine sample but also for determining semiquantitatively the concentration of the leukocytes present.

The above specification, examples, and data provide a complete description of the manufacture and the use of the many methods, compounds, compositions, and devices of the invention. Since many embodiments of the invention can be made without departing from the spirit and the scope of the invention, the invention resides in the claims hereinafter appended.